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Molecular confirmation of *Hymenolepis hibernia* in field mice (*Apodemus sylvaticus*) from St Kilda has potential to resolve a host-parasite relationship

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ABSTRACT

Insular wildlife populations provide opportunities to examine biological questions in systems that are relatively closed and potentially tractable, striking examples being the long-term studies of ecology and evolution in the red deer and feral sheep populations on the Hebridean islands of Rum and St Kilda. In the case of parasitology, Understanding of parasitic infections insular wildlife populations in conjunction with knowledge of their origins has the potential to add a fresh perspective to disease control in humans and domestic animals. In the case of parasitology, understanding infections of insular wildlife populations, in conjunction with knowledge of their origins, has the potential to add a fresh perspective to disease control in humans and domestic animals. With this in mind, gross and molecular examination for the presence of cyclophyllidean tapeworms was performed on the viscera and rectal contents of 17 preserved specimens of *Apodemus sylvaticus* field mice and on the naturally voided faeces of a further four mice on the remote archipelago of St Kilda. Molecular speciation of hexacanth embryos extracted from the faeces of two mice, using nucleotide sequence analysis of the ribosomal cytochrome c-oxidase subunit-1, confirmed infection with *Hymenolepis hibernia*. Phylogenetic analysis showed that these were genetically distinct from *Hymenolepis diminuta*, previously reported in the insular *A. sylvaticus* mice, and from other published *H. hibernia* haplotypes. There was insufficient hymenolepidid tapeworm phylogeographic variation to resolve the origins of the co-evolved St Kilda mice, primarily due to a lack of published *H. hibernia* Cox-1 sequence data across the parasite's geographical range. Nevertheless, the Maximum Likelihood haplotype tree shows the potential for molecular parasitology to resolve a host-parasite relationship once more data become available. Morphological diagnostic features of zoonotic *H. hibernia* eggs are also described.

1. Introduction

St Kilda is an uninhabited archipelago lying in the north Atlantic Ocean about 40 miles west of the Outer Hebrides, and comprising the islands of Soay, Hirta, Dun and Boreray. Hirta, the largest of these islands, with about 670 ha of exposed hill and moorland, was continuously inhabited for thousands of years. The islanders kept sheep and cattle to supplement their diet of seabirds and cereal crops and for wool to trade and pay the landlord, along with large numbers of dogs. In 1930 the human population of St Kilda was evacuated along with their animals. In 1932, 107 feral Soay sheep were relocated onto Hirta from the smaller neighbouring island of Soay, to maintain the pasture. These sheep have been left unmanaged since then, and the only other terrestrial mammals present on St Kilda have been mice and visiting humans.

Two years after the 1930 evacuation of St Kilda, the resident population of house mice (*Mus musculus*) became extinct (Harrisson and Moy-Thomas, 1933). Without the human inhabitants, these commensal animals were unable to compete with the less strongly anthropopilous field mice (*Apodemus sylvaticus*), which have survived on the islands of Hirta and Dun (Morton-Boyd, 1956; Berry, 1969; Berry and Tricker, 1969). Neither of these species would have survived the Last Glacial

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Maximum about 24 thousand years ago (Johnsen et al., 1992) in this location, hence both would have been introduced more recently, and presumably inadvertently, by humans (Corbet, 1961). The source of these animals has been a matter of considerable interest to zoologists and conservationists, with some evidence for introductions from Scandinavia that have been attributed to Vikings. However there may have been earlier and later introductions from mainland Britain or elsewhere (Berry, 1969; Jones et al., 2010; Herman et al., 2017). At various times, both the St Kilda house mouse and the field mouse have been considered as distinct species or subspecies, due to their size and pelage colour, however genetic evidence does not support any substantial differentiation from populations elsewhere in the British Isles or northern and western Europe (Jones et al., 2010; Herman et al., 2017) and this distinction consequently seems unwarranted. The St Kilda field mice survive on seeds, invertebrates and scavenged carcasses of sheep and seabirds (Berry, 1969). Previous parasitological studies have found that they are infected with adult *Hymenolepis diminuta* (Morton-Boyd, 1959) and larval metacestodes of *Taenia taeniaeformis* (referred to as *Taenia crassicolis* or cysticercus fasciolaris) (Waterston, 1906; Morton-Boyd, 1959).

The present study was undertaken to investigate the possibility that the St Kilda field mouse might be a definitive host for *Taenia hydatigena*, accounting for the high prevalence of cysticerci tenuicollis previously identified in the St Kilda Soay sheep (Torgerson et al., 1995), but this highly speculative hypothesis was not supported. Nevertheless, the study provided an opportunity for the post-mortem examination of the intestinal tracts from a series of preserved specimens of St Kilda field mice, showing the presence of adult cyclophyllidean tapeworms. In addition, examination of mouse faeces revealed the presence of taeniid eggs and permitted molecular confirmation of their species identity. The serendipitous and novel identification of *Hymenolepis hibernia* provided an opportunity to investigate the phylogenetic origins of the parasite, with reference to those of its wildlife host and to consideration of its zoonotic potential (Nkouawa et al., 2016).

2. Materials and methods

2.1. Mouse samples used to identify adult tapeworm infections

Viscera from carcasses of 17 St Kilda field mice, preserved in the vertebrate collections of the National Museums of Scotland, were examined. These had been collected between June 2011 and June 2012 from locations throughout Hirta and kept frozen at −20 °C, albeit with occasional cycles of thawing and re-freezing. Any tapeworms or tapeworm segments that were found were recovered and transferred to 70% ethanol. The rectal contents were collected for coprological examination. Naturally-voided faecal samples were also obtained opportunistically from an additional four locations in the Village Bay area on the island of Hirta.

2.2. Parasitological examination of faeces

Faecal samples were suspended in water. Sedimented material was then processed through a series of sieves. Material deposited on 53 μm and 30 μm sieves was retained and examined microscopically for the presence of cyclophyllidean cestode eggs. Eggs were picked and transferred to 70% ethanol.

2.3. Genomic DNA isolation, PCR amplification and sequence analysis of mitochondrial cytochrome c oxidase subunit-1 (Cox-1)

Aliquots of about 100 cyclophyllidean cestode eggs with hexacanth onchospheres were lysed in single 0.2 ml tubes containing 50 μl of proteinase K lysis buffer and stored at −80 °C as previously described (Redman et al., 2008). 1 μl of a 1:5 dilution of neat lysate was used as PCR template and the same dilutions of lysate buffer made in parallel were used as negative controls. A fragment of 396 bp of the mitochondrial cytochrome c oxidase subunit-1 (Cox-1), was amplified using forward (CeCox-For- TTTTTTGGGCATCCTGAGGTTTAT) and reverse primers (CeCox-Rev- TAAAGAAAGAACATAATGAAAATG) (Lavikainen et al., 2008). PCR reaction conditions consisted of 1x thermopol reaction buffer (NEB BioLab), 2 mM $MgSO_4$, 200 μM dNTPs, 0.2 μM forward and reverse primers and 1U of Phusion high fidelity DNA polymerase (Finenzyme). The thermo-cycling parameters consisted of an initial 98 °C for 30 s followed by 40 cycles of 98 °C for 10 s, 54 °C for 30 s and 72 °C for 2 min with a single final extension cycle of 72 °C for 7 min. DNA templates for direct sequencing of the Cox-1 region were cleaned using QIAquick PCR Purification Kit (Cat No./ID: 28104) following the manufacturers' protocols. Amplicons were sequenced from both ends using the same primers used for PCR amplification for each region with BigDye Terminator Cycle Sequencing (Applied Biosystems). Sequences of Cox-1 were edited to remove primers and poor quality sequence on both ends using Geneious Pro 5.4 software (Kearse et al., 2012). Sequences showing 100% base pair similarity were grouped into haplotypes using the CD-HIT Suite software (Huang et al., 2010). Haplotype and GenBank reference sequences for *Hymenolepis* spp. were imported into MEGA 6 (Tamura et al., 2013) and a Maximum Likelihood tree was inferred, using the Hasegawa-Kishino-Yano (1985) nucleotide subsitution model with gamma distribution of rates across sites (HKY + G), as selected with the Bayesian Information Criterion. Model parameters were estimated from the data and branch support was estimated from 1000 bootstrap pseudoreplicates of the data. The Maximum Likelihood tree was rooted using a closely related outgroup sequence from *H. contortus* (GenBank accession no. AF070785).

3. Results

3.1. Gross parasitological tapeworm identification

Cyclophyllidean tapeworms or segments were identified in the small intestines of five of the 17 mice that were examined postmortem. These were in poor condition and mostly fragmented, but could be separated into two morphological groups based on their segment dimensions and estimated overall length of about 2 mm wide and 6 cm long, or about 4 mm wide and up to 31 cm long. One of the smaller tapeworms had an intact, unarmed scolex (Fig. 1A), but the scolices were not found for any others. The most intact larger tapeworm, lacking a scolex, is shown in Fig. 1F. Cyclophylllidean tapeworm eggs were only recovered on the 53 μm sieve, from the faeces of each of the five mice in which adult tapeworms were found and from one of the four naturally-voided faecal samples, collected from a cleat (or cleit; an ancient small stone storage hut). Eggs in the faeces of three mice (numbered 5, 8 and 10) in which the smaller tapeworms were identified and in the faeces collected from a cleat (labelled X) were typically anoplocephalid, being approximately pyramidal-shaped, with a maximum width of 62–68 μm (Fig. 1B and D) and containing a similarly shaped onchosphere, about 38 μm across (Fig. 1E). Eggs in the faeces of the three mice (numbered 7, 8 and 9) and in the naturally-voided faeces (lettered X) were near spherical, mean 68 μm in diameter, with a thick, dark outer shell, a granular and non-striated embryophore about 20 μm deep, granular, and a thin onchospheral membrane (Fig. 1C, G, 1I and 1K) surrounding a 36–38 μm spherical hexacanth onchosphere (Fig. 1H and J and 1L). The embryonic hooks were relatively long, and consistently arranged in offset, radiating pairs. Cyclophyllidean co-infections were, therefore, identified in mouse 8 and in the naturally-voided faeces from mouse X. However, the lack of non-overlapping and definitive morphological diagnostic characters for cyclophyllidean eggs and the poor condition of the adult tapeworms prevented identification to generic or specific level.

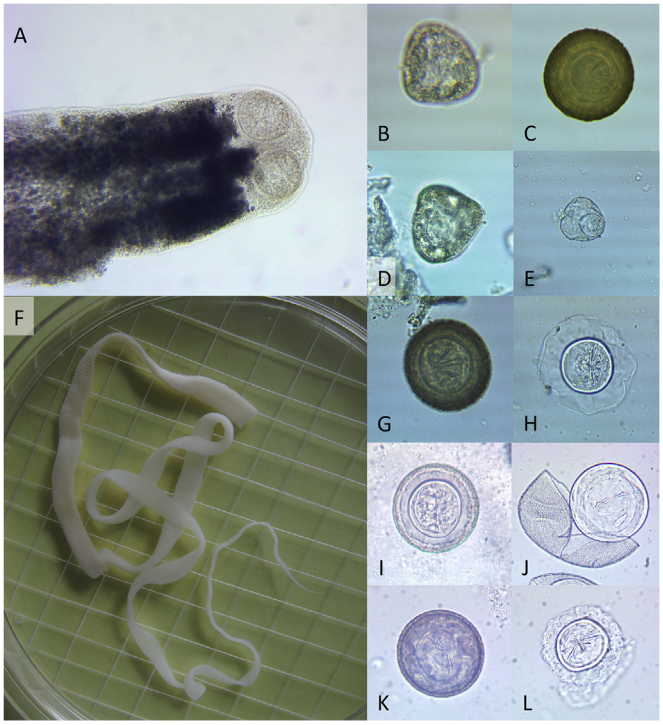

Fig. 1. Examples of cyclophyllidean tapeworms and eggs recovered from *Apodemus sylvaticus* viscera and faeces. A: Unarmed scolex of an intact tapeworm - mouse 5. B: Anoplocephalid tapeworm egg - mouse 8. C: Hymenolepidid egg cropped without changing dimensions from the same image as B, for comparison - mouse 8. D: Anoplocephalid tapeworm egg - mouse X (faeces from a cleat). E: Anoplocephalid onchosphere released after squashing an egg under a cover slip - mouse X (faeces from a cleat). F: Tapeworm from mouse 8 (scolex not intact). G: hymenolepidid tapeworm egg - mouse X (faeces from a cleat). H: Hexacanth onchosphere surrounded by an onchospheral membrane and inner zone of the embryophore, which has swollen, having been released from the egg shell by squashing under a cover slip - mouse 9. I: Hymenolepidid tapeworm egg - mouse 7. J: Hexacanth onchosphere surrounded by an intact onchospheral membrane and inner and outer zones of the embryophore, being released from a cracked egg shell by squashing under a cover slip - mouse 7. K: Hymenolepidid tapeworm egg - mouse 8. L: Hexacanth onchosphere surrounded by an onchospheral membrane and inner zone of the embryophore, which has swollen, having been released from the egg shell by squashing under a cover slip - mouse 8.

3.2. Confirmation of H. hibernia in St Kilda mice by analysis of mt-Cox-1 sequences

385 bp mt-Cox-1 fragments of cyclophyllidean DNA were amplified and sequenced from eggs recovered from mouse 9 and faeces X (despite repeated attempts, DNA did not amplify from the eggs containing hexacanth onchospheres recovered from mice 7 and 8). The fragments were aligned with 34 sequences of four *Hymenolepis* species (*H. Hibernia*, *H. diminuta*, *Hymenolepis nana* and *Hymenolepis microstoma*) published in the NCBI Genbank database. The St Kilda sequences were identical and comparison with the previously published *H. hibernia* sequences revealed that they formed a distinct haplotype, characterised by 12 intraspecific SNPs within the 385 bp fragment of the mt-Cox-1 locus (Fig. 2). The 31 *H. diminuta*, 6 *H. hibernia*, 2 *H. microstoma* and 9 *H. nana* mt-Cox-1 sequences from Genbank comprise of 19, 5, 2 and 8 unique haplotypes, respectively. In the Maximum Likelihood tree inferred from the 35 haplotypes, including the new one from St Kilda, there are four well-supported monophyletic clades, comprising the *H. diminuta*, *H. hibernia*, *H. microstoma* and *H. nana* sequences (Fig. 3). The two rodent-origin species, *H. diminuta* and *H. hibernia*, and the two human-origin species, *H. microstoma* and *H. nana*, form distinct clades in the phylogenetic tree. The *H. hibernia* clade is highly supported (bootstrap percentage 100) and includes the new haplotype from the St Kilda mice (H-HA25). The St Kilda *H. hibernia* sequence is nevertheless distinct and clusters with an eastern European haplotype (H-HA20), separately from haplotypes of distant origins in Asia (H-HA21, 22, 23 and 24).

4. Discussion

The tapeworm species *H. diminuta* was previously reported in the *A. sylvaticus* mouse population from St Kilda (Morton-Boyd, 1959). However, species identification of helminth parasites by morphology can be challenging, not least in the case of *Hymenolepis sensu lato* (Makarikov and Tkach, 2013; Binkienė et al., 2018). Biochemical, growth and behavioural studies suggest that *H. hibernia* and *H. diminuta* are genetically distinct, with *H. hibernia* being adapted to Palaearctic mice from the genus *Apodemus*, whereas *H. diminuta* is a rat tapeworm (Montgomery et al., 1987). The molecular evidence provided by our study supports the suggestion (Montgomery et al., 1987) that *H. diminuta* and *H. hibernia* are genetically distinct. It also suggests that the former species may previously have been wrongly identified as the latter on St Kilda, and potentially elsewhere, as a result of their overlapping morphological characteristics. Similar genetic differentiation has been shown between *H. diminuta* and a cryptic species, closely related to *H. hibernia*, that was recovered from a Tibetan woman (Nkouawa et al., 2016).

The presence of *H. hibernia* in field mice from St Kilda is of potential interest with regard to the origins of both the parasite itself and its co-evolved host (Wilson et al., 1998). The presence of a glacial refugium for *A. sylvaticus* in northern Europe was previously predicted from the distribution of mitochondrial genetic variation in its nematode parasite *Heligmosomoides polygyrus*, and this was subsequently confirmed from genetic variation in *A. sylvaticus* itself (Nieberding et al., 2004, 2005; Herman et al., 2017). However, there was insufficient resolution in the Maximum Likelihood tree (Fig. 2) for this approach to be effective in the present case, which may to some extent reflect the limited variation present in the 385 bp mt-Cox-1 fragment, although it is most likely due to insufficient sampling across its geographical range. Nevertheless, genetic variation in *H. hibernia* may become sufficiently informative in the future, once more molecular data become available. The sensitivity of the approach could be improved by cloning and sequencing to identify all of the haplotypes present in a mixed pool of eggs, albeit the number of haplotypes present in clonally reproducing cyclophyllidean tapeworms would be predicted to be small. Other approaches such as the use of polymorphic microsatellite markers (Pajeulo et al., 2015), or of next generation sequencing methods to describe the phylogenetic origins of multiple parasite species derived from the same host (Avramenko et al., 2015) might also provide future direction.

Molecular speciation methods have proved to be sensitive and unambiguous in situations where complementary phenotypic characterisation has already been undertaken (Wimmer et al., 2004). Precise species identification is important in the study of parasite life histories and epidemiology that is required to define control strategies. *Hymenolepis senso latu* including the rodent-origin species *H. hibernia* (Nkouawa et al., 2016) and *H. diminuta* (Hancke and Suarez, 2017) are zoonotic, hence informed preventive management is necessary. The basic life history of hymenolepidid tapeworms involves incidental human and definitive rodent hosts being infected when they ingest cysticercoid metacestodes with beetle intermediate hosts, although autoinfection with eggs can also occur in humans. It is therefore important that further studies are made of hymenolepidid tapeworm phylogeographic variation and epidemiology.

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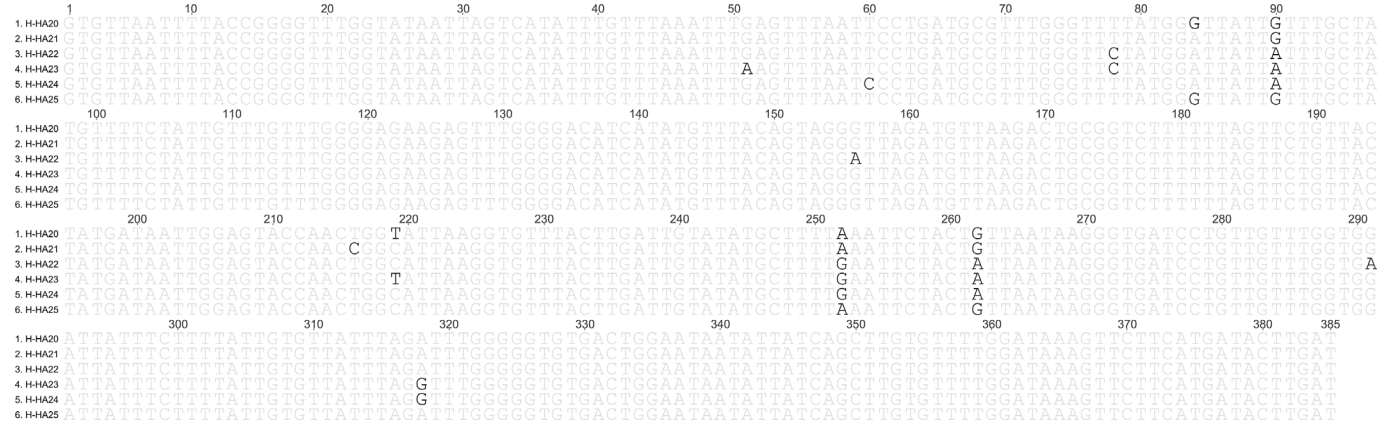
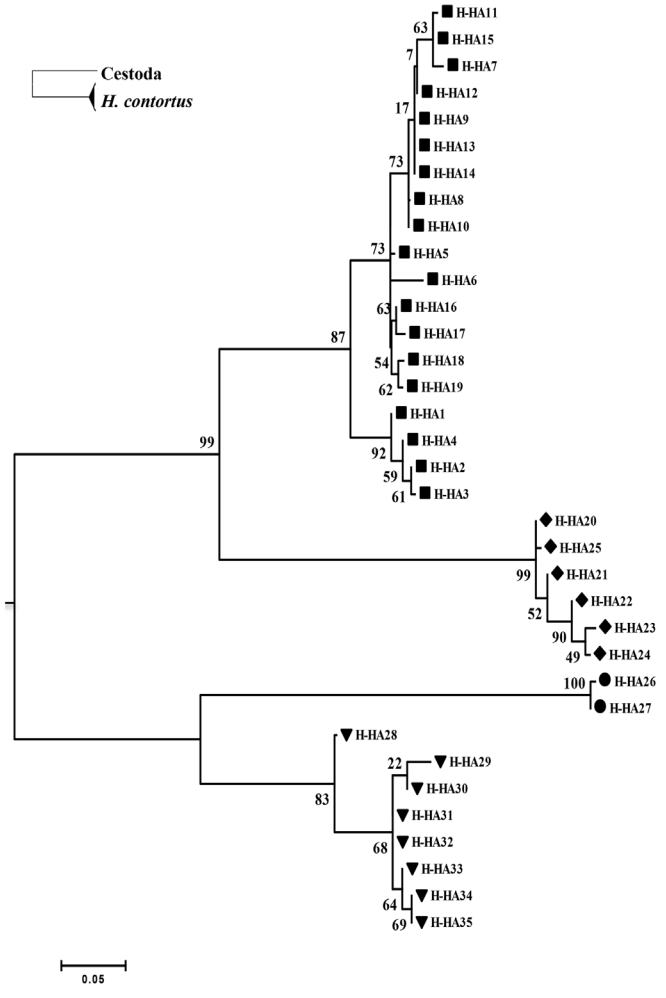


Fig. 2. The Sanger sequence of 385 bp of mt-Cox-1 fragments of cyclophyllidean DNA generated from eggs recovered from mouse 9 and faeces X (6. H-HA25), aligned with 5 corresponding *H. hibernia* sequences published in the NCBI Genbank database. There are 12 intraspecific SNPs at positions 5, 60, 78, 84, 90, 156, 216, 219, 252, 262, 291 and 318. GeneBank submission ID: 2151861.



Tree label	Accession number	*Hymenolepis* species	Host	Country of origin
H-HA11	AB494469	*H. diminuta*	*Rattus norvegicus*	Japan
H-HA15	AP017664	*H. diminuta*	Unknown	Denmark
H-HA15	LM387386	*H. diminuta*	Unknown	Denmark
H-HA15	LC063185	*H. diminuta*	Unknown	Laboratory strain
H-HA15	KC990403	*H. diminuta*	Laboratory rat	USA
H-HA15	KF689686	*H. diminuta*	*Rattus norvegicus*	Poland
H-HA15	KC990402	*H. diminuta*	Laboratory rat	USA
H-HA7	AY121843	*H. diminuta*	Rat	Australia
H-HA12	LC063186	*H. diminuta*	*Rattus rattus*	Spain, Canaries
H-HA9	JN258044	*H. diminuta*	*Rattus norvegicus*	Spain, Tenerife
H-HA9	JN258042	*H. diminuta*	*Rattus norvegicus*	Spain, La Palma
H-HA9	JN258043	*H. diminuta*	*Rattus rattus*	Spain, Gran Canaria
H-HA13	AF314223	*H. diminuta*	Unknown	Laboratory strain
H-HA14	KC990401	*H. diminuta*	Laboratory rat	USA
H-HA14	KF689687	*H. diminuta*	Unknown	Poland
H-HA8	JN258048	*H. diminuta*	*Rattus norvegicus*	Spain, Tenerife
H-HA10	JN258047	*H. diminuta*	*Rattus rattus*	Spain, La Gomera
H-HA5	KP317829	*H. diminuta*	Ring-tailed lemur	China
H-HA5	KP317831	*H. diminuta*	Ring-tailed lemur	China
H-HA5	KP317830	*H. diminuta*	Ring-tailed lemur	China
H-HA6	LC063184	*H. diminuta*	*Rattus rattus*	Madagascar
H-HA16	JN258045	*H. diminuta*	*Rattus rattus*	Spain, Fuerteventura
H-HA17	KY462776	*H. diminuta*	*Rattus rattus*	South Africa
H-HA17	KY462777	*H. diminuta*	*Rattus rattus*	South Africa
H-HA17	KY462775	*H. diminuta*	*Rattus rattus*	South Africa
H-HA18	KY462778	*H. diminuta*	*Rattus norvegicus*	South Africa
H-HA19	JN258046	*H. diminuta*	*Rattus rattus*	Spain, Lanzarote
H-HA1	KY079337	*H. diminuta*	*Rattus norvegicus*	China
H-HA4	AB494470	*H. diminuta*	*Rattus norvegicus*	Japan
H-HA2	KY079338	*H. diminuta*	*Rattus norvegicus*	China
H-HA3	AF096244	*H. diminuta*	Rat	Republic of Korea
H-HA20	LC063172	*H. hibernia*	*Apodemus flavicollis*	Croatia
H-HA20	LC063173	*H. hibernia*	*Apodemus agrarius*	Romania
H-HA25		*H. hibernia*	*Apodemus sylvaticus*	St Kilda Mouse 9
H-HA25		*H. hibernia*	*Apodemus sylvaticus*	St Kilda Mouse X
H-HA21	LC063174	*H. hibernia*	*Apodemus uralensis*	Kazakhstan
H-HA22	LC063177	*H. hibernia*	*Apodemus agrarius*	South Korea
H-HA23	LC063179	*H. hibernia*	*Apodemus agrarius*	South Korea
H-HA24	LC063178	*H. hibernia*	*Apodemus agrarius*	South Korea
H-HA26	LC063188	*H. microstoma*	*Mus musculus*	Japan
H-HA27	JN258051	*H. microstoma*	*Mus musculus*	Spain
H-HA28	AY121842	*H. nana*	Unknown	Unknown
H-HA29	HM447234	*H. nana*	*Homo sapiens*	Mexico
H-HA30	GU433103	*H. nana*	Rat	Egypt
H-HA31	GU433104	*H. nana*	*Homo sapiens*	Egypt
H-HA32	KY079336	*H. nana*	*Rattus norvegicus*	China
H-HA33	AB494472	*H. nana*	*Mesocricetus auratus*	Uruguay
H-HA34	AB494471	*H. nana*	*Mus musculus*	Japan
H-HA35	LC063187	*H. nana*	*Mus musculus*	Japan
H-HA35	AB033412	*H. nana*	Unknown	Japan

Fig. 3. Maximum Likelihood tree for the 35 haplotypes identified from 39 mt-cox-1 sequences of *Hymenolepis* species. Of these, 19, 5, 2 and 8 haplotypes are identified in the Genbank database as *H. diminuta*, *H. hibernia*, *H. microstoma* and *H. nana*, respectively, and one haplotype (H-HA25) was identified here from the faeces of St Kilda mice 9 and X. Branches with bootstrap values (1000 replications) represented at the base of the nodes. The phylogeny is rooted with mt-cox-1 sequence of parasitic nematode *H. contortus*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2018.09.007.

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